

Dephosphorylation of Phytate from Rice Bran and Soybean Meal Using Phytases from *Aspergillus* sp. 5990

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Total phosphorus contents in rice bran and soybean meal were determined to be 5.81 and 2.77%, respectively, and 97.2% of phosphorus in rice bran and 66.4% in soybean meal were presented as phytate phosphorus. Optimum pH condition for hydrolysis of phytate in rice bran and soybean was determined to be in the pH range of 3.7 and 5.3. The highest activity of phytase for hydrolysis of phytate in both samples was determined to be at 55°C for rice bran and 55-60°C for soybean. Hydrolysis of phytate in soybean meal at pH 5.0 increased with the co-reaction or consecutive reaction with protease; however, in rice bran hydrolysis decreased with co-reaction with protease. Phytate degradation of soybean meal in the presence of pepsin at pH 2.5 showed higher than that of rice bran. Phytate degradation of rice bran in the presence of trypsin or pancreatin at pH 7.0 increased the activity around 2-times compared with the activity in the absence of trypsin or pancreatin. The results of this study suggest that hydrolysis of phytate in rice bran or soybean meal with phytase and protease may provide an alternative process for the preparation of aquacultural feed with a low level of organic phosphorus.

Key words: *Aspergillus* sp. phytase, Protease, Rice bran, Soybean meal

Introduction

Phytate (*myo*-inositol 1,2,3,4,5,6-hexakisdihydrogen phosphate) is the major storage form of phosphate in plants, comprising 1-5% by weight of edible legumes, cereals, oil seeds, pollen, and nuts (Cheryan, 1980). Monogastric animals lack or have low phytase activities in their digestive system, and most undigested phytate are excreted in their manure. The presence of phytate in monogastric animal feeds is an antinutritional factor, since the phosphate moieties of phytate chelate essential minerals such as Ca²⁺, Zn²⁺, Mg²⁺, Fe²⁺, and potentially other trace minerals (Erdman, 1979; Maga, 1982). Another problem is the high levels of undigested phytate in the fecal waste, which can be discharged in the sewage and become a primary cause of algal blooms in water environments.

Rice bran and soybean meal contains approximately 30 and 70%, respectively, of protein and have been

widely used as a feedstuff in feed industry. To reduce high feed cost, some aquaculture farmers have used rice bran and soybean meal as a feed supplement for protein source. However, a high phytate level in rice bran and soybean meal may impede mineral utilization and excreted organic phosphate from animal waste may cause algal blooms. Excretion of phytate phosphorus by monogastric animals such as poultry, swine, and fish can result in water pollution (Raboy, 2001). The most common response to nutrient loading in water environment is the formation of blooms of phytoplankton, some of which can be noxious or toxic to fishes or invertebrates. High concentrations of nitrogen and phosphorus contamination in aquatic ecosystem occurred around industrial animal production area (Mallin, 2000). In coastal America, water pollution is a major problem because fish-kills are taking place in the warm water of Chesapeake Bay and oxygen-depleted "Red Zones" are showing up in Gulf of Mexico (Ullah et al., 2000).

One promising technique to enhance digestion of

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organic phosphorus is phytase supplements in animal feeds. This technique can result in significant decreases in the amount of phosphorus excreted by livestock (Mallin, 2000). However, application of phytase in aquaculture feeds has several limitations. Body temperature of fish is influenced by environmental temperature of fish habitation. Generally, water temperature at aquaculture farms in Korea is around 12-25°C, in which temperature is too low to exert catalytic activity of phytase compared to body temperature of terrestrial animals. Furthermore, digestion time of phytate in fish intestine is too short to hydrolyze phytate to inorganic phosphates and myo-inositol derivatives. An alternative method to decrease phytate level in aquacultural feeds is an enzymatic treatment of feed supplement with phytase. It has been reported that 66% of phytate phosphorus in the barley diet and 73% of that in the wheat diet were degraded by a treatment of microbial phytase at 38°C for 2 hr. (Dorthe and Poulsen, 2003)

In this study, degradation conditions of phytate were determined with crude phytases from *Aspergillus* sp. 5990 to reduce phytate phosphorus in rice bran and soybean meal, and effect of pepsin, trypsin or pancreatin on phytate degradation by phytase was examined.

Materials and Methods

Raw materials

Soybean meal was donated from Taehan Sugar Co. (Seoul, Korea). Before using for phytase reaction, soybean meal was pulverized to less than 100 mesh in a local milling plant (Yosu, Korea). Rice bran was supplied by a local rice milling plant (Suncheon, Korea).

Enzyme preparation

Aspergillus sp. 5990 has been isolated from soil near the root of leguminous plants (*Glycine* spp.) as described by Kim et al. (1999). Fungus was cultured in a medium containing 1% sucrose, 0.2% (NH₄)₂SO₄, 0.3% tryptone, 0.2% yeast extract, 0.05% KCl, 0.05% MgSO₄, 0.001% MnSO₄·5H₂O, 0.001% FeSO₄, and 0.1% triton X-100. Cultivation was carried out for 3 days in a 7 L laboratory bioreactor (KoBioTek, Korea) under constant cultivation conditions. Temperature was held at 35°C and pH was not regulated. After fermentation, culture supernatant was collected after centrifugation at 12,000×g for 20 min and stored at -20°C.

Phytase activity

Phytase activity was determined by a modified method of Greiner et al. (1993). The reaction mixture containing 10 µL of enzyme solution, 250 µL of 5 mM sodium phytate (pH 5.0), and 1 mM CaCl₂ was incubated at 37°C for 30 min. The reaction was stopped by adding 1.5 mL of a freshly prepared solution of acetone:5 N H₂SO₄:10 mM ammonium molybdate (2:1:1, v/v/v) and 100 µL of 1.0 M citric acid to the assay mixture. The liberated inorganic phosphate was measured by a modification of the ammonium molybdate method. Any cloudiness was removed by centrifuging at 3,000×g for 15 min prior to the measurement of the absorbance at 410 nm. Quantification was based on a standard curve generated with a 0-4 µM sodium monobasic phosphate standard. The phytase activity (U) was expressed as 1 µmol phosphate liberated per min per mL of enzyme solution at 37°C.

Determination of phytate

Phytate from rice bran and soybean meal was extracted by a modified method of Harland and Oberleas (1977). Five grams of rice bran or soybean meal were stirred with 100 mL of 0.65 N HCl at room temperature and centrifuged at 3,000×g for 20 min. The supernatant was applied onto an AG1-X8 anion-exchange column (1×5 cm, Cl⁻ form, 200-400 mesh, SIGMA) and eluted with 15 mL of 0.1 M NaCl solution to remove inorganic phosphorous as well as other interfering compounds. The phytate was eluted with 15 mL of 0.7 M NaCl solution and made up to 100 mL with distilled water. An aliquot (1.0 mL) of the eluent was mixed with 3.0 mL of Wade reagent (0.03% FeCl₃·6H₂O in sulfosalicylic acid), and the solution was mixed on a vortex mixer for 5 sec. The absorbance of supernatant after centrifugation at 8,000×g for 10 min was measured at 500 nm and quantified by comparing a standard curve prepared with various concentration of phytate.

Proximate analysis

Total nitrogen (TN) was determined by a semimicro Kjeldahl method. Ash, fat, and moisture contents were measured by standard methods (AOAC, 1995).

Determination of phytase concentration

Ten grams of rice bran or soybean meal were suspended in 50 mL of 0.1 M sodium acetate buffer, pH 5.5, and placed in a shaking water bath maintained at 37°C. A different amount of phytase (0-2.0 U/g) was added in each reaction mixture and incubated

for 30 min. After the reaction, 5 mL of reaction mixture was transferred to a test tube and the reaction was stopped by adding 5 mL of 20% trichloroacetic acid (TCA) solution and centrifuged for 20 min at 3,000×g. Aliquots of supernatant were determined for the assay of inorganic phosphorus.

Determination of pH and temperature dependence

One gram of rice bran or soybean meal was suspended in 5 mL of various pH buffer solutions and placed in a shaking water bath maintained at 37°C. One U of phytase for rice bran and 0.3 U of phytase for soybean meal were added in each test tube and incubated for 30 min with shaking. After the reaction, 5 mL of 20% trichloroacetic acid (TCA) solution was added and centrifuged for 20 min at 3,000×g. Aliquots of supernatant were taken for the assay of inorganic phosphorus. Optimum temperature was determined with 1.0 U of phytase for rice bran and 0.3 U for soybean meal at pH 5.0 for 30 min reaction at different temperatures (20-65°C).

Statistical analysis

All data were the mean of 5 replicates. Statistical analysis was performed by analysis of variance (ANOVA) and Duncan's multiple range tests ($p < 0.05$) using SAS statistical software (SAS Inst. 1996).

Results and Discussion

General composition

Total phytate phosphorus in rice bran and soybean meal comprised 5.65 and 1.84%, respectively (Table 1). Almost all phosphorus in rice bran existed as phytate phosphorus (82.9%), however, soybean meal showed 66.4% of phytate phosphorus. The different values found in the literature on the content of phytate phosphorus of feedstuffs are related to cultivars, processing conditions, and analytical methods used

Table 1. General composition of rice bran and soybean meal¹ (%)

Component	Rice bran	Soybean meal
Moisture	15.6±0.70	12.3±0.65
Ash	7.75±0.47	6.21±0.45
Crude lipid	18.3±0.55	2.43±0.44
Crude protein	29.6±1.19	44.7±2.67
Phosphorus		
Phytate phosphorus	5.65±0.24	1.84±0.14
Free phosphorus	0.16±0.01	0.93±0.10

¹Analyzed based on dry matter.

(Viveros et al., 2000).

Rice bran had the highest level of phytate phosphorus, being about 6.55-6.83% in cereals and legumes as well as their processed feedstuffs (Phillippy, 2003). Higher total and phytate phosphorus content of rice bran than rice can be explained by the abundance of phytate phosphorus in the protein rich aleurone layer of the rice, as compared with corn, where about 90% of the phytate is located in the germ portion (O'Dell et al., 1972). Soybean meal had about 66.4% of the total phosphorus in the form of phytate phosphorus, and this result is similar to that obtained by other researchers (Viveros et al., 2000; Phillippy, 2003).

Effect of enzyme concentration

High level of phytate phosphorus needs to be degraded to inorganic phosphorus prior to use in feed formulation to facilitate absorption of phosphorus in the intestine of fish. As shown in Fig 1, the amount of released inorganic phosphorus from rice bran was increased with the amount of phytase up to 1.0 U, however, 0.3 U of phytase was highest for hydrolysis of phytate in soybean meal. Therefore, phytase concentrations for hydrolysis of phytate were established to be 1.0 U for rice bran and 0.3 U for soybean meal.

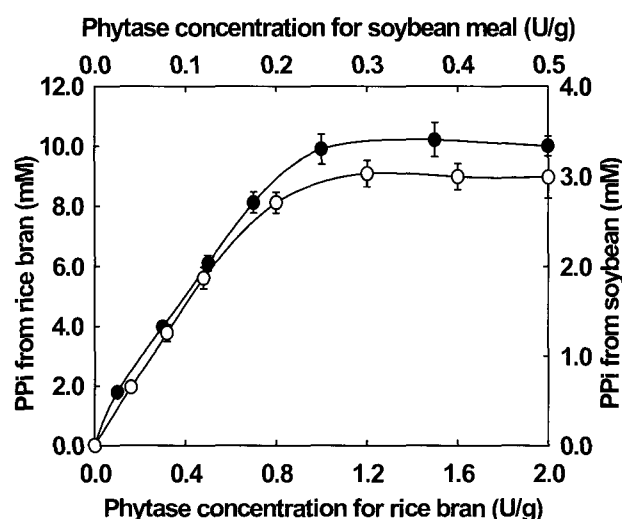


Fig. 1. Effect of phytase concentration on hydrolysis of phytate in rice bran (●) and soybean meal (○). Reactions were performed at pH 5.0 and 37°C.

The amount of commercial phytase recommended in the feed industry is approximately 0.5 U per gram feed (Viveros et al., 2002). The level of phytase concentration for hydrolysis of phytate phosphorus

in rice bran and soybean meal was similar to commercial use of phytase for monogastric animals (Wodzinski and Ullah, 1996).

Effect of pH and temperature

Effects of pH on hydrolysis of phytate were performed using 1 U/g of rice bran and 0.3 U/g of soybean meal. As shown in Fig. 2, degradation of phytate was highest in pH range of 3.7-5.3 in both samples.

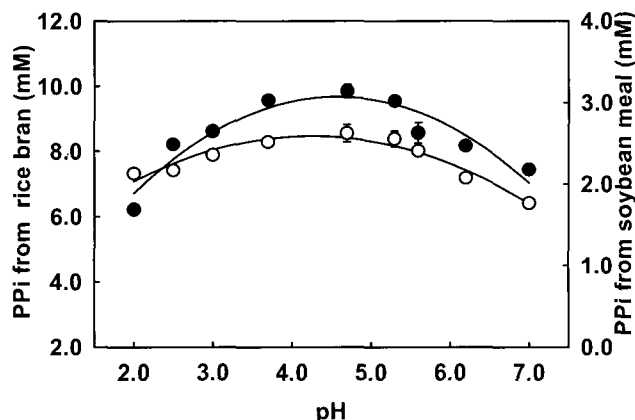


Fig. 2. Effect of pH on hydrolysis of phytate in rice bran (●) and soybean meal (○). Reactions were performed at 37°C and indicated pH with 0.1 U for rice bran and 0.03 U for soybean meal.

Optimum pH for degradation of phytate by crude phytase from *Aspergillus* sp. 5990 was different from that with purified phytase (Kim et al., 1999). This difference resulted from the combined activity of phytase and acid phosphatase in *Aspergillus* sp. (Kim et al., 1999; Ullah and Gibson, 1987). Two distinct pH optima have been commonly identified in the phytases from *Aspergillus* sp. (Ullah and Gibson, 1987; Wyss et al., 1999b; Pasamontes et al., 1997), however, those were not shown on the degradation of phytate in soybean meal and rice bran used as substrates.

Optimum temperature of hydrolysis of phytate in rice bran and soybean meal was determined with 1.0 U/g for rice bran and 0.3 U/g for soybean meal at pH 5.0 for 30 min incubation. The highest degradation of phytate was displayed at 55°C for rice bran and at 55-60°C for soybean meal (Fig. 3). Therefore, optimum reaction conditions for hydrolysis of phytate in rice bran and soybean meal were determined to be pH 5.5 and 50°C with 1.0 U/g for rice bran and 0.3 U/g for soybean meal.

Several criteria must be met for the commercializa-

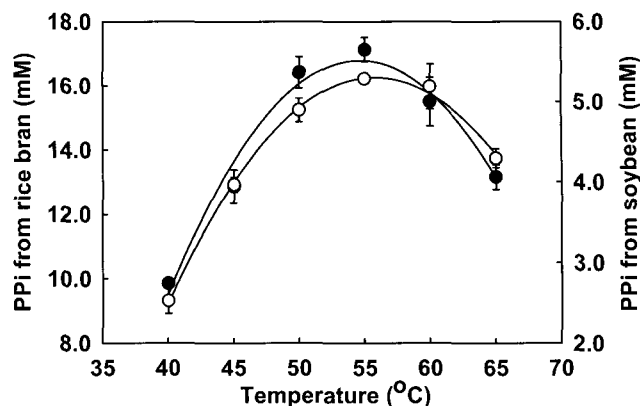


Fig. 3. Effect of temperature on hydrolysis of phytate in rice bran (●) and soybean meal (○). Reactions were performed at pH 5.0 and indicated temperature with 0.1 U for rice bran and 0.03 U for soybean meal.

tion of phytase in the animal feed industry. It should be thermostable during pelletization and resistant to protease in the digestive tract, and it should have broad substrate specificity with a high specific activity. Thermal stability of phytase is relevant in animal feed applications, where the enzyme is normally incorporated into the feedstuff prior to pelletization and the feed mixture exposed to processing temperatures of 85 to 90°C for short periods of time. In this circumstance a commercial phytase must be able to withstand brief heating prior to ingestion by the animal. Optimum temperature of phytase from *Aspergillus* sp. 5990 was determined to be at 65°C (Kim et al., 1999), however, that of the phytase in this study was identified as 55°C for rice bran and 55-60°C for soybean meal as a substrate. The result in this study showed lower optimum temperature than the previous study, where activity was determined with sodium phytate as a substrate.

Effect of protease on degradation of phytate

Phytate in cereal or legumes is distributed as phytin, which is a complex of phytate with proteins and minerals. A moiety of protein in phytin may be attacked by digestive proteases such as pepsin, trypsin, and pancreatin in the digestive tract. Degradation of protein residues in phytin would facilitate the action of phytase. As shown in Table 2, relative hydrolytic activity of phytase against phytin in rice bran at pH 5.0, 2.5, and 7.0 were determined to be 100, 75, and 40%, respectively.

In the case of co-reaction with phytase and protease, the level of phytase activity at pH 5.0 was reduced by about 10% of original activity with reaction in

Table 2. Effect of protease on degradation of phytin in rice bran at different pHs (%)

Reaction pH	Control	Co-reaction with protease			Consecutive reaction with protease		
		Pepsin	Trypsin	Pancreatin	Pepsin	Trypsin	Pancreatin
pH 5.0	100	91.5±2.26 ^a	97.3±2.12 ^b	89.6±2.39 ^a	99.7±2.78 ^a	101±2.43 ^a	101±2.98 ^a
pH 2.5	75	78.6±2.16	ND	ND	80.5±1.78	ND	ND
pH 7.0	40	ND	89.9±2.55 ^a	71.5±3.16 ^b	ND	79.2±2.16 ^a	79.0±2.12 ^a

Co-reaction was performed with 10 g of rice bran, 10 U of phytase and 10 U of each protease at indicated pH for 60 min. Consecutive reaction was performed with 10 g of rice bran and 10 U of protease for 30 min and then reaction was repeated for 30 more min after adding 10 U of phytase at indicated pH. ND, not determined.

Table 3. Effect of protease on degradation of phytin in soybean meal at different pHs (%)

Reaction pH	Control	Co-reaction with protease			Consecutive reaction with protease		
		Pepsin	Trypsin	Pancreatin	Pepsin	Trypsin	Pancreatin
pH 5.0	100	111±3.81 ^a	109±2.55 ^a	104±2.04 ^b	106±2.82 ^a	113±2.56 ^b	111±2.55 ^a
pH 2.5	75	84.6±3.52	ND	ND	87.2±2.55	ND	ND
pH 7.0	40	ND	73.4±2.84 ^a	71.4±2.55 ^a	ND	70.1±2.26 ^a	81.1±2.30 ^b

Co-reaction was performed with 10 g of soybean meal, 3.0 U of phytase and 10 U of each protease at indicated pH for 60 min. Consecutive reaction was performed with 10 g of soybean meal and 10 U of protease for 30 min and then reaction was repeated for 30 more min after adding 3.0 U of phytase at indicated pH. ND, not determined.

the presence of pepsin or pancreatin; however, trypsin did not affect phytase activity. This indicates that that pepsin and pancreatin would cause degradation of phytase during reaction at pH 5.0. Phytase activity at pH 2.5 with pepsin increased by 3.6% of original activity compared with phytase alone, which suggests that pepsin did not contribute to the degradation of the phytase at pH 2.5. However, phytase activity at pH 7.0 in the presence of trypsin or pancreatin increased to 2.25-fold with trypsin and 1.79-fold with pancreatin compared with phytase alone. Phytase activity incorporated with trypsin or pancreatin at pH 7.0 accelerated the degradation of phytin, which suggests that phytin in rice bran would be much more easily degraded in the digestive tract.

There is a possibility that proteases hydrolyze phytase during co-reaction. As an alternative, phytin degradation was performed in a two step process. Firstly, protein incorporated in phytin was degraded with protease for 30 min and then phytin was hydrolyzed with phytase for 30 min. In consecutive reaction with proteases and phytase, phytase activity at pH 5.0 was not affected by any protease treatments. Phytase activity at pH 2.5, after pepsin treatment, increased 3.5% of original activity. Furthermore, phytin degradation at pH 7.0, after trypsin or pan-

creatin treatment, increased to 2-times compared with phytase alone. The consecutive reaction of phytase after protease treatment showed higher degradation of phytin in rice bran at pH 5.0 and pH 2.5.

Co-reaction with phytase and pepsin, trypsin or pancreatin at pH 5.0 increased degradation of phytin 4-11% in soybean meal (Table 3). The result showed proteases improved phytase activity at pH 5.0. Phytase activity at pH 2.5 with pepsin increased by 9.6% of original activity compared with phytase alone. This suggests that pepsin contributed to the phytase activity at pH 2.5 by degrading protein. Also, phytase activity at pH 7.0 in the presence of proteases increased to 1.83-fold with trypsin and 1.79-fold with pancreatin compared with phytase alone. Phytase activity after incorporation of trypsin or pancreatin at pH 7.0 accelerated the degradation of phytin, which suggests that phytin in soybean meal would be much more easily degraded in the digestive tract.

Consecutive reaction with proteases and phytase at pH 5.0 showed a similar result as the co-reaction. Degradation of protein in phytin by protease treatment facilitated the phytase action on the degradation of phytate. Furthermore, phytase activity at pH 2.5 after pepsin treatment increased 12.2%. Phytin degradation at pH 7.0 after trypsin or pancreatin treatment in-

creased 1.75- or 2.03-fold, respectively, compared with phytase alone. The difference of phytin degradation between trypsin and pancreatin treatment might be explained by the presence of trypsin inhibitor (Olli et al., 1994).

A. niger phytase was stable at acid pH range and retained 95% of its activity after pepsin treatment (Phillippy, 1999). The most likely explanation for the greater resistance of *A. niger* phytase to proteolysis was its higher level of glycosylation (Wyss et al., 1999a). Like pepsin stability, *A. niger* phytase was stable to pancreatin (Phillippy 1999). The stability of the phytase against proteolytic enzymes would be a good explanation for the improved phytase activity in the presence of proteases. The result in this study provides evidence that protease may help phytin degradation when it is combined with protein, which results in more exposure of phytin to phytase. Exposed phytin will be more susceptible to phytase and might increase hydrolytic rate of phytin by phytase.

Phytase activity functions in the stomach because commercial phytase has an optimum pH at pH 2.5 and 5.0. However, pepsin does not completely hydrolyze food proteins in the stomach and phytin would be bound with protein, which will impede phytase activity. As seen in Tables 2 and 3, phytase activity at intestinal pH (around pH 7.0) was low compared to its optimum pH (5.0), thus, phytase could successfully exert its catalytic activity with an assist from trypsin in the intestine. Therefore, phytases from *Aspergillus* sp. 5990 might be potential candidates for the degradation of phytin for aquaculture feed-stuffs since they have higher resistance to digestive proteases.

Acknowledgments

This work was supported by Pukyong National University Research Fund in 2000.

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(Received March 2004, Accepted June 2004)